

**APPLICATION FOR UNITED STATES
LETTERS PATENT**

**METHODS FOR IDENTIFICATION, ASSESSMENT, PREVENTION, AND
THERAPY OF CANCER**

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METHODS FOR IDENTIFICATION, ASSESSMENT, PREVENTION, AND THERAPY OF CANCER

Field of the Invention

The field of the invention is cancer, including diagnosis, characterization, and therapy of carcinoma.

Background of the Invention

The increased number of cancer cases reported in the United States, and, indeed, around the world, is a major concern. Currently there are only a handful of treatments available for specific types of cancer, and these provide no absolute guarantee of success. Among them, ovarian cancer is the fifth most common cancer (other than skin cancer) in women. It ranks fifth as the cause of cancer death in women. The American Cancer Society estimates that there will be about 25,580 new cases of ovarian cancer in this country in 2004. About 16,090 women will die of the disease.

Despite advances in the chemotherapy, surgery and supportive care, death rates for this disease have remained constant for nearly two decades (National Cancer Institute. SEER Cancer. Statistics Review 1973-1997, 2001). New diagnostic methods and therapies are thus needed.

SUMMARY OF THE INVENTION

The invention relates to the newly identified cancer therapeutic targets (hereinafter “targets” or “targets of the invention”), which are listed in Table 1. These targets are overexpressed in carcinomas generally, and more specifically to adenocarcinoma and squamous cell carcinoma, including colon, breast, lung, ovary, cervix, and prostate cancers. Table 1 provides the sequence identifiers of the sequences of such marker nucleic acids and proteins listed in the accompanying Sequence Listing.

The invention also relates to the cancer markers (hereinafter “markers” or “markers of the invention”), which are listed in Tables 1. These markers are overexpressed in carcinomas generally, and more specifically to adenocarcinoma and squamous cell carcinoma, including colon, breast, lung, ovary, cervix, and prostate cancers. The invention provides nucleic acids and proteins that are encoded by or correspond to the markers (hereinafter “marker nucleic acids” and “marker proteins”, respectively). Tables 1 provide the sequence identifiers of the sequences of such marker nucleic acids and proteins listed in the accompanying Sequence Listing.

The invention also relates to various methods, reagents and kits for diagnosing, staging, prognosing, monitoring and treating carcinoma, including ovarian cancer. In one embodiment, the invention provides a diagnostic method of assessing whether a patient has carcinoma or has higher than normal risk for developing carcinoma, comprising the steps of comparing the level of expression of a marker of the invention in a patient sample and the normal level of expression of the marker in a control, e.g., a sample from a patient without carcinoma. A significantly higher level of expression of the marker in the patient sample as compared to the normal level is an

indication that the patient is afflicted with carcinoma or has higher than normal risk for developing carcinoma.

In a preferred diagnostic method of assessing whether a patient is afflicted with carcinoma (e.g., new detection ("screening"), detection of recurrence, reflex testing), the method comprises comparing:

- a) the level of expression of a marker of the invention in a patient sample, and
- b) the normal level of expression of the marker in a control non-cancerous sample.

A significantly higher level of expression of the marker in the patient sample as compared to the normal level is an indication that the patient is afflicted with carcinoma.

The invention also provides diagnostic methods for assessing the efficacy of a therapy for inhibiting carcinoma in a patient. Such methods comprise comparing:

- a) expression of a marker of the invention in a first sample obtained from the patient prior to providing at least a portion of the therapy to the patient, and
- b) expression of the marker in a second sample obtained from the patient following provision of the portion of the therapy.

A significantly lower level of expression of the marker in the second sample relative to that in the first sample is an indication that the therapy is efficacious for inhibiting carcinoma in the patient.

It will be appreciated that in these methods the "therapy" may be any therapy for treating carcinoma including, but not limited to, chemotherapy, radiation therapy, surgical removal of tumor tissue, gene therapy and biologic therapy such as the administering of antibodies and

chemokines. Thus, the methods of the invention may be used to evaluate a patient before, during and after therapy, for example, to evaluate the reduction in tumor burden.

In a preferred embodiment, the diagnostic methods are directed to therapy using a chemical or biologic agent. These methods comprise comparing:

- a) expression of a marker of the invention in a first sample obtained from the patient and maintained in the presence of the chemical or biologic agent, and
- b) expression of the marker in a second sample obtained from the patient and maintained in the absence of the agent.

A significantly lower level of expression of the marker in the second sample relative to that in the first sample is an indication that the agent is efficacious for inhibiting carcinoma, in the patient. In one embodiment, the first and second samples can be portions of a single sample obtained from the patient or portions of pooled samples obtained from the patient.

The invention additionally provides a monitoring method for assessing the progression of carcinoma in a patient, the method comprising:

- a) detecting in a patient sample at a first time point, the expression of a marker of the invention;
- b) repeating step a) at a subsequent time point in time; and
- c) comparing the level of expression detected in steps a) and b), and therefrom monitoring the progression of the carcinoma in the patient.

A significantly higher level of expression of the marker in the sample at the subsequent time point from that of the sample at the first time point is an indication that the carcinoma has

progressed, whereas a significantly lower level of expression is an indication that the carcinoma has regressed.

The invention further provides a diagnostic method for determining whether carcinoma has metastasized or is likely to metastasize in the future, the method comprising comparing:

- a) the level of expression of a marker of the invention in a patient sample, and
- b) the normal level (or non-metastatic level) of expression of the marker in a control sample.

A significantly higher level of expression in the patient sample as compared to the normal level (or non-metastatic level) is an indication that the carcinoma has metastasized or is likely to metastasize in the future.

The invention moreover provides a test method for selecting a composition for inhibiting carcinoma in a patient. This method comprises the steps of:

- a) obtaining a sample comprising cancer cells from the patient;
- b) separately maintaining aliquots of the sample in the presence of a plurality of test compositions;
- c) comparing expression of a marker of the invention in each of the aliquots; and
- d) selecting one of the test compositions which significantly reduces the level of expression of the marker in the aliquot containing that test composition, relative to the levels of expression of the marker in the presence of the other test compositions.

The invention additionally provides a test method of assessing the carcinogenic potential of a product. This method comprises the steps of:

- a) maintaining separate aliquots of cells in the presence and absence of the product; and

b) comparing expression of a marker of the invention in each of the aliquots.

A significantly higher level of expression of the marker in the aliquot maintained in the presence of the product, relative to that of the aliquot maintained in the absence of the product, is an indication that the product possesses carcinogenic potential. An example of a known carcinogenic product that increases the risk of ovarian cancer is lysophosphatidic acid.

In addition, the invention further provides a method of inhibiting carcinoma in a patient. This method comprises the steps of:

- a) obtaining a sample comprising cancer cells from the patient;
- b) separately maintaining aliquots of the sample in the presence of a plurality of compositions;
- c) comparing expression of a marker of the invention in each of the aliquots; and
- d) administering to the patient at least one of the compositions which significantly lowers the level of expression of the marker in the aliquot containing that composition, relative to the levels of expression of the marker in the presence of the other compositions.

In the aforementioned methods, the samples or patient samples comprise cells obtained from the patient. The cells may be found in tumor biopsies.

Definitions

A "marker" is a gene whose altered level of expression in a tissue or cell from its expression level in normal or healthy tissue or cell is associated with a disease state, such as cancer. A "marker nucleic acid" is a nucleic acid (e.g., -mRNA, siRNA, cDNA, oligonucleotides) encoded by or corresponding to a marker of the invention. Such marker nucleic acids include DNA (e.g.,

cDNA, oligonucleotides) comprising the entire or a partial sequence of any of the nucleic acid sequences set forth in the Sequence Listing or the complement of such a sequence. The marker nucleic acids also include RNA comprising the entire or a partial sequence of any of the nucleic acid sequences set forth in the Sequence Listing or the complement of such a sequence, wherein all thymidine residues are replaced with uridine residues. A "marker protein" is a protein encoded by or corresponding to a marker of the invention. A marker protein comprises the entire or a partial sequence of any of the sequences set forth in the Sequence Listing. The terms "protein" and "polypeptide" are used interchangeably.

The "normal" level of expression or amount of a marker is the level of expression or amount of the marker in cells of a human subject or patient not afflicted with carcinoma. An "over-expression" or "significantly higher level of expression" of a marker refers to an expression level in a test sample that is greater than the standard error of the assay employed to assess expression, and is preferably at least twice, and more preferably three, four, five or ten times the expression level of the marker in a control sample (e.g., sample from a healthy subjects not having the marker associated disease) and preferably, the average expression level of the marker in several control samples.

A "significantly lower level of expression" of a marker refers to an expression level in a test sample that is at least twice, and more preferably three, four, five or ten times lower than the expression level of the marker in a control sample (e.g., sample from a healthy subject not having the marker associated disease) and preferably, the average expression level of the marker in several control samples.

According to the invention, the level of expression or amount of a marker of the invention in a sample can be assessed, for example, by detecting the presence in the sample of: the corresponding marker protein (e.g., a protein having one of the sequences set forth as "SEQ ID NO (2, 4, 6 and 8)" in Table 1, or a fragment of the protein (e.g. by using a reagent, such as an antibody, an antibody derivative, an antibody fragment or single-chain antibody, which binds specifically with the protein or protein fragment).

The corresponding marker nucleic acid (e.g. a nucleotide transcript having one of the nucleic acid sequences set forth as "SEQ ID NO 1, 3, 5 and 7" in Table 1, or a complement thereof, or a fragment of the nucleic acid, e.g. by contacting transcribed polynucleotides obtained from the sample with a substrate having affixed thereto one or more nucleic acids having the entire or a segment of the nucleic acid sequence of any of the SEQ ID NO 1, 3, 5 and 7, or a complement thereof, a metabolite which is produced directly (i.e., catalyzed) or indirectly by the corresponding marker protein.

According to the invention, any of the aforementioned methods may be performed using a plurality (e.g. 2, 3, or more) of cancer markers, including epithelial or other cancer markers known in the art. In such methods, the level of expression in the sample of each of a plurality of markers, at least one of which is a marker of the invention, is compared with the normal level of expression of each of the plurality of markers in samples of the same type obtained from control humans not afflicted with carcinoma. A significantly altered (i.e., increased or decreased as specified in the above-described methods using a single marker) level of expression in the sample of one or more markers of the invention, or some combination thereof, relative to that marker's corresponding normal or control level, is an indication that the patient is afflicted with

carcinoma. For all of the aforementioned methods, the marker(s) are preferably selected such that the positive predictive value of the method is at least about 10%.

The methods of the invention have the following uses:

- (1) assessing whether a patient is afflicted with carcinoma;
- (2) assessing the presence of cancer cells;
- (3) making antibodies, antibody fragments or antibody derivatives that are useful for treating cancer and/or assessing whether a patient is afflicted with cancer;
- (4) making the DNA fragment including but not restricting the primers, antisense nucleotides, siRNA that are useful for treating cancer and/or assessing whether a patient is afflicted with cancer;
- (5) assessing the efficacy of one or more test compounds for inhibiting cancer in a patient;
- (6) assessing the efficacy of a therapy for inhibiting cancer in a patient;
- (7) monitoring the progression of cancer in a patient;
- (8) selecting a composition or therapy for inhibiting cancer in a patient;
- (9) treating a patient afflicted with cancer;
- (10) inhibiting cancer in a patient;
- (11) assessing the carcinogenic potential of a test compound; and
- (12) preventing the onset of cancer in a patient at risk for developing cancer.

Detailed description of the invention

The invention relates to cancer markers associated with the cancerous state of ovarian cells. It has been discovered that the higher than normal level of expression of any of these markers or combination of these markers correlates with the presence of ovarian cancer. Methods are provided for detecting the presence of ovarian cancer in a sample, the absence of ovarian cancer in a sample, the stage of ovarian cancer, and other characteristics of ovarian cancer that are relevant to prevention, diagnosis, characterization, and therapy of ovarian cancer in a patient. The methods of the present invention may similarly apply to detecting the presence of other cancer in a sample, the absence of cancer in a sample, the stage of cancer, and other characteristics of cancer that are relevant to prevention, diagnosis, characterization, and therapy of cancer in a patient.

It is a simple matter for the skilled artisan to determine whether a marker is overexpressed in ovarian cancer cells. For example, expression of a marker of the invention may be assessed by any of a wide variety of well known methods for detecting expression of a transcribed nucleic acid or protein. In a preferred embodiment, expression of marker is assessed using the Real-Time Quantitative RT-PCR. By preparing the ovary RNA from the patients with or without ovarian cancer, the Real-Time Quantitative RT-PCR will be performed through the following protocol using the marker specific primer pairs as listed in Table2 and the Sequence Listing. In brief, first-strand cDNA was synthesized at 50 °C for 60 min, followed by a 10-min denaturation at 95 °C using the proper RT-PCR enzyme kit. PCR reactions were then performed in the same tubes using the following conditions for 40 cycles: 95 °C for 30 s, 60 °C for 30 s, and 68 °C for 60 s.

In another preferred embodiment, immunological methods also could be used to determine the overexpression of a marker of the invention. Using the antibody which specifically recognize the protein of the markers, the skilled artisan could detect the expression of the marker in tissue sample or protein extraction from the patients with or without ovarian cancer. The antibody is derived from the full length protein or short peptide.

The level of expression of a marker in normal (i.e. non-cancerous) human ovarian tissue can be assessed in a variety of ways. In one embodiment, this normal level of expression is assessed by assessing the level of expression of the marker in a portion of ovarian cells which appears to be non-cancerous and by comparing this normal level of expression with the level of expression in a portion of the ovarian cells which is suspected of being cancerous. Alternately, and particularly as further information becomes available as a result of routine performance of the methods described herein, population-average values for normal expression of the markers of the invention may be used. In other embodiments, the 'normal' level of expression of a marker may be determined by assessing expression of the marker in a patient sample obtained from a non-cancer-afflicted patient, from a patient sample obtained from a patient before the suspected onset of ovarian cancer in the patient, from archived patient samples, and the like.

To determine whether a target of the invention is a therapeutic target for ovarian cancer, the skilled artisan could inhibit the RNA expression of the targets then detect the survival rate of the ovarian cancer cells. The methods are described as follow. In brief, about $0.75 \sim 2 \times 10^4$ /cm² ovarian cancer cells (such as TOV-112D cells) are incubated at 37°C in wells containing growth medium (such as TOV-112D cells; 1:1 mixed medium of MCDB 105 {Sigma-Aldrich, MO, USA} and Medium 199 {Life Technologies, Inc., MD, USA} supplemented with 15% calf

serum {Life Technologies, Inc., MD, USA}) under a 5% (v/v) CO₂, 95% air atmosphere. The cells are then transfected using a standard transfection mixture comprising 200 nM of target specific siRNA (such as SEQ ID 17, SEQ ID 18) and 2 microliters of OligofectamineTM (Invitrogen Corporation, CA, USA) per well. The cells are incubated in the transfection mixture for about 5 hours, and then replaced with fresh growth medium. After 48 hr, the cell survival rate was determined by adding MTT (Sigma-Aldrich, MO, USA) to the cell cultures at a final concentration of 1 mg/ml. And after 5 hr incubation at 37°C, the dark crystals formed were dissolved in DMSO and the cell viability was indicated by the amount of crystals which was obtained by measuring the absorbance of the solution at 570/630 nm. The lower survival rate in the target specific siRNA treated cells will be revealed by comparing the survival rate in the cells which treated with negative control siRNA.

Materials and Methods:

Cell Lines and Tissue Samples. The human ovarian papillary serous cystadenocarcinoma cell line, OC 314, was obtained from the ICLC Animal Cell Lines Database (Servizio Biotecnologie IST, Centro di Biotecnologie, Avanzate L.go R. Benzi, 10, 16132 Genova, Italia). The cells were propagated in RPMI 1640 medium (Life Technologies, Inc., -MD, USA) supplemented with 5% calf serum (Life Technologies, Inc., MD, USA) and 2 mM L-glutamine (Sigma-Aldrich, MO, USA). The other human cell lines including TOV-112D (derived from ovarian endometrioid carcinoma), TOV-21G (derived from ovarian clear cell carcinoma), CC7T/VGH (derived from cervical carcinoma), H184B5H5/M10 (human mammary epithelial cell), T/G HA-VSMC (normal aorta smooth muscle cell) and HFL 1 (lung fibroblast)

were obtained from Food Industry Research and Development Institute (331 Shih-Pin Road, Hsinchu, 300 Taiwan R.O.C.). TOV-112D and TOV-21G cells were propagated in the 1:1 mixed medium of MCDB 105 (Sigma-Aldrich, MO, USA) and Medium 199 (Life Technologies, Inc., MD, USA) supplemented with 15% calf serum (Life Technologies, Inc., MD, USA). CC7T/VGH cells were propagated in DMEM (Life Technologies, Inc., MD, USA) supplemented with 10% calf serum (Life Technologies, Inc., MD, USA). H184B5H5/M10 cells were propagated in GIBCO 11900 medium (Life Technologies, Inc., MD, USA) supplemented with 10% calf serum (Life Technologies, Inc.). T/G HA-VSMC cells were propagated in the Ham's F12K medium (HyClone Inc., Logan, UT, USA) supplemented with 10% calf serum (Life Technologies, Inc., MD, USA), 0.05 mg/ml ascorbic acid (Life Technologies, Inc., MD, USA), 0.01 mg/ml insulin (Sigma-Aldrich, MO, USA), 0.01 mg/ml transferrin (Sigma-Aldrich, MO, USA), 10 ng/ml sodium selenite (Sigma-Aldrich, MO, USA), and 0.03 mg/ml endothelial cell growth supplement (Sigma-Aldrich, MO, USA). HFL 1 cells were propagated in Ham's F12K medium (HyClone Inc., UT, USA) supplemented with 10% calf serum (Life Technologies, Inc., MD, USA).

The total RNA of human normal ovary (Catalog number: CR0856) and human ovary tumor (Catalog number: 64011-1) were purchased from Clontech (CA, USA).

Microarray: Two human oligo microarray chips (H04 and H05) were constructed from the oligolibrary of Human Release 1.0 (Compugen Inc., Tel Aviv, Israel). A total of 18861 oligo-probes were presented on these two arrays.

0.25 µg of total RNA of each sample is reversed transcribed into cDNA and further in vitro transcribed into cRNA and labeled with CyDye using Amino Allyl MessageAmp aRNA Kit (Ambion, Texas, USA) according to the manufacturer protocol. cRNA of sample normal ovary was labeled with Cy3 and acts as the reference sample. cRNA of sample ovary tumor was labeled with Cy5 and acts as the experimental sample. 1.5 µg of each labeled aRNA of reference and experimental sample was purified, combined, and mixed 2X hybridization buffer according to the manufacturer protocol before applied on the microarray.

Hybridization was done in dark at 38.5°C for 16 hours. Hybridization and washing conditions were followed according to the manufacturer protocol of CyScribe First-Strand cDNA Labeling Kit (Amersham Biosciences, England).

Microarray image was scanned using GenePix® 4000B microarray scanner (Axon Instruments, Inc, CA, USA). Image was acquired and analyzed using GenePix® Pro 4.1 software (Axon Instruments, Inc, CA, USA). Image was quality checked and lowess normalized using GeneData Expressionist Refiner v3.0 software (GeneData AG, Basel, Switzerland).

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) assays: Total RNA was extracted from each cell sample using TRI REAGENT (Molecular Research Center, Inc., Ohio, USA) according to the manufacturer protocol. Purified RNA was treated with RNase-free DNase I (Ambion, Texas, USA) to remove residual genomic DNA contamination following the manufacturer's protocol. cDNA synthesis and quantitative real-time RT-PCR was performed using the TITANIUM One-Step RT-PCR kit (Clontech, Palo Alto, CA, USA) containing SYBR Green I (BioWhittaker Molecular Applications; BMA, Rockland, ME, USA). In brief, first-strand cDNA was synthesized at 50 °C for 60 min, followed by a 10-min

denaturation at 95 °C. PCR reactions were then performed in the same tubes using the following conditions for 40 cycles: 95 °C for 30 s, 60 °C for 30 s, and 68 °C for 60 s. The sequences of primers are listed in Table 2 and Sequence Listing. RT-PCRs were performed in triplicate for each RNA sample for both the gene of interest (target gene) and the reference gene (beta-actin). Real-time fluorescence monitoring and melting curve analysis were performed using Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Negative controls containing no DNA template were included in each experiment. A melting curve was created at the end of PCR cycle to confirm that a single product was amplified. Data were analyzed by Rotor-Gene 3000 operating software version 4.6.94 (Corbett Research) to determine the threshold cycle (C_T) above the background for each reaction. The relative transcript amount of the target gene, calculated using standard curves of serial RNA dilutions, was normalized to that of beta-actin of the same RNA.

RNA interfering: siRNA oligonucleotides were designed for targeting the sequence of IRTKS (5'-AAGCACUGUGGCUUUGCAAAC-3') and Solt (5'-AACACUCACCGAUUCAAAUGC-3'). The target sequence (AATTCTCCGAACGTGTCACGT) which has 16 base overlap with *Thermotoga maritima* section 21 of 136 of the complete genome was used as a negative control siRNA. siRNAs were synthesized by the silencerTM siRNA Construction Kit (Ambion, Texas, USA) following the manufacturer's protocol. siRNA transfection were performed in 24-well plates using the OligofectamineTM (Invitrogen Corporation, CA, USA), LipoFectamineTM 2000 (Invitrogen Corporation, CA, USA), or siPORTTM Amine (Ambion, Texas, USA), depending on the cell types.

Cell viability assay: The cell viability was determined by adding MTT (Sigma-Aldrich, MO, USA) to the cell cultures at a final concentration of 1 mg/ml. After 5 hr incubation at 37°C, the dark crystals formed were dissolved in DMSO and the amount was obtained by measuring the absorbance of the solution at 570/630 nm.

We found that the selected 4 genes were up-regulated in ovarian cancer tissue and cell lines (normal ovary and ovarian cancer RNA were purchased from BD Biosciences Clontech) using microarray and Quantitative real-time RT-PCR methods. These genes are listed in Table 1 and Sequence Listing.

Moreover, using the ovarian cell lines as the cell model, we found that inhibiting the expression of IRTKS or Solt could decrease the growth of ovarian cancer cells based on our RNAi experiment. Since IRTKS is one of the insulin receptor tyrosine kinase substrate and Solt is transcription factor related protein, both of them should be involved in the signal transduction pathway of cell growth or development. Based on our finding, IRTKS and Solt are potential therapeutic targets for ovarian cancer.

Thus, while there have shown and described and pointed out fundamental novel features of the invention as applied to a preferred embodiment thereof, it will be understood that various omissions and substitutions and changes in the form and details of the devices illustrated, and in their operation, may be made by those skilled in the art without departing from the spirit of the invention. For example, it is expressly intended that all combinations of those elements and/or method steps which perform substantially the same function in substantially the same way to achieve the same results are within the scope of the invention. Moreover, it should be

recognized that structures and/or elements and/or method steps shown and/or described in connection with any disclosed form or embodiment of the invention may be incorporated in any other disclosed or described or suggested form or embodiment as a general matter of design choice. It is the intention, therefore, to be limited only as indicated by the scope of the claims appended hereto.